Development of a Homogenous and Label-less AlphaScreen® Based Platform to Measure MEK2 Activity

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1 Abstract

Mitogen-activated protein kinases, MAPKs, are constituents of numerous signal transduction pathways, and are activated by protein kinase cascades. There is great interest in finding compounds that target components of the MAPK pathways as possible treatment for inflammatory diseases and cancer. Here we report the development of a novel AlphaScreen® assay to measure MEK2 mediated phosphorylation of Erk-1. Following a microformated *in vitro* enzymatic reaction, GSH (glutathione) coated donor beads were used in combination with PhosphoSensor acceptor beads to capture the phosphorylated GST-Erk-1 protein substrate and, as a result, generate an AlphaScreen signal proportional to the amount of phosphorylated product. The assay was shown to be very sensitive, using as low as 30 nM of GST-Erk1 substrate and 1 nM of MEK2 kinase. Under these conditions, excellent S/B and Z'-factors could be obtained. We have thus developed a highly sensitive and versatile label-less assay platform that can serve as a kinase substrate deorphanization tool. This platform is unique as it allows direct measurements of kinase activities on full-length protein substrates where no antibody is available and is easily amenable to automation and HTS.

2 Introduction

Deregulation of protein phosphorylation has been associated with many human diseases leaving kinases at the forefront of drug discovery. This is reflected by the number of FDA approved drugs currently on the market as well as the large amount of compounds presently at different stages of clinical trials.

One of the major goals of HTS is to efficiently discover compounds that act on a specific target with which to start medicinal chemistry. For HTS applications, biochemical in vitro kinase assays represent useful tools for measuring kinase activity. The majority of those biochemical assays take advantage of the availability of small peptide substrate to measure kinase activity. However, the use of short peptide substrates could present major disadvantages. Firstly, from the approximately 500 kinases encoded by the human genome, some enzymes may not show any activity unless a full length substrate is used in vitro. Secondly, allosteric modulators of kinases may only be monitored by using full length protein as substrate. Lastly, the use of peptide substrates is far from being physiologically relevant, thus may be misleading when assessing compound potency.

This presentation demonstrates the use of a label-less AlphaScreen application to monitor the phosphorylation of full length GST-Erk1 by MEK2. This assay takes advantage of using the PhosphoSensor Acceptor beads in conjunction with the newly developed Glutathione Donor beads (GSH coated Donor beads). This HTS compatible assay highlights the advantages of AlphaScreen to measure the phosphorylation of full length proteins without the need for time consuming protein modification or expensive antibodies.

3 AlphaScreen MEK2 Kinase Assay

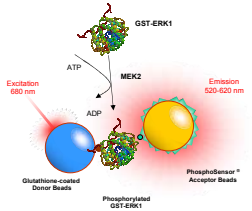

Figure 1. AlphaScreen kinase activity detection. Following GST-Erk1 phosphorylation by MEK2, the GSH Donor and PhosphoSensor Acceptor beads will be brought into close proximity. Following laser excitaiton of the Donor beads (in blue) at 680 nm, singlet oxygen will be produced that will travel up to 200 nm and activate a series of energy transfer inside the Acceptor beads (in yellow). The activated Acceptor beads will then re-emit light between 520 and 620 nm. In the absence of phosphorylation, the beads will not come into proximity and no signal will be emitted.

4 Materials and Methods

Titration of Inhibitors

2 µL MEK2
2 µL serial dilutions of inhibitors
Incubate 15 min at RT
2 µL ATP/ GST-Erk1 mix
Incubate 1 hour at RT
2 µL EDTA (10mM final in 8 µl reaction)
Incubate 15 min at RT
9 µL Phosphosensor Acceptor beads (20 µg/mL final in 26 µL reaction)
9 µL GSH-Donor beads (20 µg/mL final in 26 µL reaction)
Incubate 1h and overnight at RT
Read on Envision Alpha Reader

- Kinase reaction was performed in the following buffer: 25 mM Hepes pH 7.4, 100 mM NaCl, 0.5 mM $MgCl_2$, 1 mM DTT, 0.01% Tween-20
- Detection reaction was performed in the following buffer: 20 mM Tris-HCl pH 7.0, 100 mM NaCl, 0.1% Tween-20

Z'- factor study

5 µL MEK2
5 µL staurosporine (10 µM final in 15 µL reaction)
Incubate 15 min at RT
5 µL ATP/ GST-Erk1 mix
Incubate 1 hour at RT
5 µL of mix of GSH-Donor beads (20 µg/mL) and EDTA (10 mM final in 20 µL reaction)
Incubate 15 min at RT
20 µL Phosphosensor Acceptor beads (20 µg/mL final in 40 µL reaction)
Incubate 1h and overnight at RT
Read on Envision Alpha Reader

- Kinase reaction was performed in the following buffer: 25 mM Hepes pH 7.4, 100 mM NaCl, 0.5 mM $MgCl_2$, 1 mM DTT, 0.01% Tween-20
- Detection reaction was performed in the following buffer: 20 mM Tris-HCl pH 7.0, 100 mM NaCl, 0.1% Tween-20

5 ATP and GST-Erk1 Substrate Titration

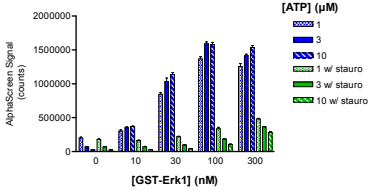

Figure 2. Determination of optimal ATP and GST-Erk1 substrate concentrations. Titration of ATP and GST-Erk1 was performed in the presence and in the absence of the generic inhibitor, staurosporine. The concentration of MEK2 was fixed at 3 nM. Detection time was overnight. The optimal concentrations of ATP and inactive GST-Erk1 were determined to be 10 µM and 30 nM, respectively. Using these assay conditions a signal to background (S/B) ratio of 26.3 was observed.

6 MEK2 Titration

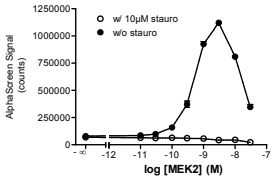

Figure 3. Determination of the optimal MEK2 concentration. MEK2 was titrated in the presence and in the absence of staurosporine. The assay was performed using 30 nM substrate (GST-Erk1), and 10 µM ATP and kinase and detection reactions were incubated 2h and overnight, respectively. The optimal concentration of MEK2 was determined to be between 1 nM and 3 nM. The sensitivity of the assay allows for the detection of substrate phosphorylation using as low as 100 pM of enzyme. Further experiments were performed using 30 nM of substrate and 3 nM of enzyme.

7 Titration of ATP

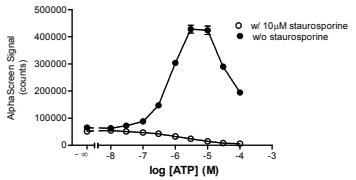

Figure 4. Determination of the optimal concentration of ATP in the kinase reaction. Titration of ATP was performed in the presence and in the absence of the generic inhibitor staurosporine. In this experiment ATP titration was performed using 3 nM MEK2 and 30 nM GST-Erk1. The concentration of ATP generating the optimal window was determined to be between 3 and 10 µM.

8 EDTA Optimization

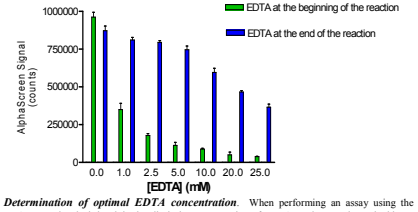

Figure 5. Determination of optimal EDTA concentration. When performing an assay using the Phosphosensor Acceptor beads, it is advised to limit the concentration of EDTA used to terminate the kinase reaction. In this experiment EDTA titration was performed using 3 nM MEK2, 10 µM ATP and 30 nM of GST-Erk1, in the presence of 0.5 mM $MgCl_2$. To confirm termination of the kinase activity, EDTA was also added at the beginning of the enzymatic reaction. 10 mM of EDTA was determined suitable for stopping the kinase reaction, while leaving a S:B of approximately 50.

9 Time Course of Enzymatic and Detection Reactions

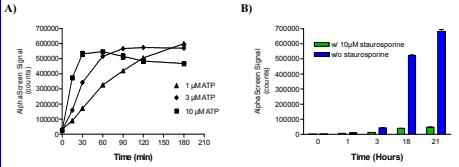

Figure 6. Time course of the enzymatic and detection reactions. A) The time course of MEK2 activity was monitored over time. The experiment was performed in the presence of 3 nM MEK2, 30 nM GST-Erk1, and 1, 3 and 10 µM ATP. The kinase reaction was terminated using 10 mM EDTA. The optimal incubation time for an assay containing 3 nM MEK2, 1 µM ATP, and 30 nM GST-Erk1was determined to be 1 hour. B) Time course of the detection reaction. The experiment was performed in the presence of 3 nM MEK2, 1 µM ATP and 30 nM GST-Erk1. A proportional increase in the signal to background ratio was observed as a function of time with maximum signal achieved overnight (18 and 21 hrs).

10 Inhibitor Potencies

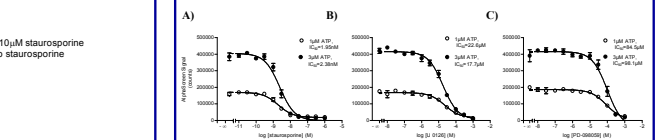

Figure 7. Inhibition of MEK2 activity with A) the generic inhibitor staurosporine, B) the specific non-competitive MEK inhibitors U0126, and C) PD-098059. This experiment was performed using 3 nM MEK2, 1 µM ATP, 30 nM GST-Erk1, and a 1 hour kinase reaction. Staurosporine was the most potent inhibitor of MEK2 activity, whereas PD-098059 was the least efficient inhibitor. U0126 and PD-098059 all appear to be non-competitive with respect to ATP. These findings are in agreement with what has been reported in the literature. As expected, PD-098059 resulted in a less potent inhibition of MEK2 than the U0126 compound.

Ref.: 1- Favata, M.F. *et al.* (1998) J. Biol. Chem. 273: 18623-18632.

2- Dudley, D.T. *et al.* (1995) Proc. Natl. Acad. Sci. U.S.A. 92: 7686-7689.

11 Z'- factor Study

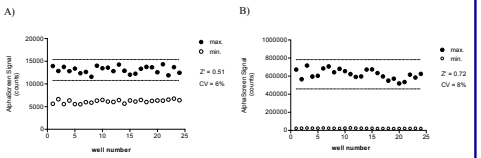

Figure 8. Determination of the suitability of the MEK2 kinase assay for HTS. The experiment was performed using 3 nM MEK2, 1 µM ATP, 30 nM GST-Erk1, and a kinase reaction time of 1 hour. Detection time was A) 1 hour and B) overnight. The minimum signal was determined in the presence of 10 µM staurosporine. After a 1 hour detection reaction a signal to background ratio (S/B) of 2.1 and CV values of 6% were observed. After an overnight detection an S/B ratio of 27.1 and CV values around 8% were obtained.

12 In Summary

- Assay development of a HTS compatible assay that allows, without cumbersome protein labeling, to measure the activity of MEK2 on its natural full length substrate, Erk1, was demonstrated.
- The assay generated windows of approximately 30 with CVs under 10% (manually dispensed), which allow the generation of Z'-values of 0.72, substantially over the widely accepted value of 0.5 for an HTS assay.
- Although overnight incubations were required to obtain an optimal window, Z'-value of 0.5 could also be generated following a 1 hr incubation of the detection reaction, due to the low assay variability.
- Evaluation of inhibitors under screening assay conditions confirmed the potency reported by other teams using alternative technologies.